THE LOCALIZATION OF ACTIN-LIKE FIBERS IN CULTURED NEUROBLASTOMA CELLS AS REVEALED BY HEAVY MEROMYOSIN BINDING

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INTRODUCTION

Actin-like protein has been isolated from cultured sympathetic ganglia of chick (6) and mammalian brain (18), and accounts for up to 20% of the total cell protein (6). The exact ultrastructural localization of such a large amount of actin-like material is essential in order to interpret its function in the motile processes of nerve cells. The *in situ* distribution of actin-like fibers in glycerinated models of nonmuscle cells was first demonstrated by the elegant experiments of Ishikawa et al. (12). This technique was based on Huxley's (11) demonstration that F-actin forms characteristic arrowhead complexes (decorated filaments) when treated with heavy meromyosin (HMM). Since then other investigators have utilized this technique to demonstrate the existence of actin-like components in a variety of cell types (1, 4, 7, 9, 16). Chick embryonic nerve cells contain actin-like fibers which interact with HMM (12); however, since normal cell morphology is disrupted during glycerination, the exact localization of actin-HMM complexes in axons and cell bodies has not been ascertained.

We have used an established line of murine neuroblastoma cells which are capable of differentiating into neurons when placed upon substrates. The axons formed by the cells may reach several millimeters in length and contain 250-Å microtubules, 100-Å filaments, and dense-core granules (15, 20, 21; J. Rosenbaum, personal communication). Other nervelike properties of these cells include their ability to generate action potentials in response to acetylcholine or electrical stimulation (10, 14) and their production of enzymes which synthesize and metabolize neurotransmitters (2, 3, 17). In this report, we describe a shorter glycerination procedure which preserves overall nerve cell shape and thus permits the ultrastructural

localization of actin-like protein in all parts of the neuroblastoma cell. The results demonstrate that actin-like protein is present along the entire length of axons and that it may be involved in axoplasmic flow.

MATERIALS AND METHODS

The murine neuroblastoma C-1300 (Clone Neuro-2a) cells used in this study were a gift from Dr. Joel Rosenbaum (for properties of this clone, see reference 13). Cells were grown in Falcon tissue culture dishes (Falcon Plastics, Division of BioQuest, Oxnard, Calif.) containing Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N.Y.), supplemented with 10% newborn calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin, and were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. Rabbit HMM was prepared according to the methods of Szent-Gyorgyi (22) and Pollard et al. (16). Glycerination was carried out at room temperature. Cells were first treated with 50% glycerol in standard salt solution (0.05 M KCl, 0.005 M $MgCl_2$, and 0.006 M potassium phosphate buffer, pH 7.0) (12) for 1–2 h, followed by 25% glycerol in standard salt for 1–2 h. They were then reacted with HMM (1–2 mg/ml) in 5% glycerol-standard salt solution for 2–4 h. Controls were treated exactly the same as experimentals, except that no HMM was present in the final step. Glycerol extracted controls, experimentals, and unglycerinated neuroblastoma cells were prepared for electron microscope studies as described previously (8). Flat-embedded cells with axons longer than 25 μm were selected with an inverted microscope, marked with a Leitz micromanipulator, cut out with a razor blade, and remounted for thin sectioning. Thin sections were cut parallel to the substrate side of the cell.

RESULTS

Differentiated neuroblastoma cells possess one or more long axons and numerous fine filopods which project both from the cell body and along the length of the axon (Fig. 1). In the electron microscope, cells not treated with glycerol contain microtubules, filaments, and microfilaments in cell bodies and axons. Microfilaments (40–60 Å diameter) are numerous in the filopod projections, but few are seen along the length of axons (Fig. 3) or in the cell bodies.

Most of the glycerinated cells remain attached to the substrate and retain their normal morphology (Fig. 2). Electron microscopy also demonstrates that the overall shape of the nerve cells is retained after glycerination (Fig. 4). At higher magnification, the cell body is seen to contain masses of actin-HMM complexes localized just beneath the plasma membrane. A thick layer of these complexes oriented parallel to each other and to the cell membrane is found on the side of the cell in contact with its substrate (Fig. 5). Arrowhead configurations could be seen frequently (Fig. 5 *a*). The upper cell surface (away from the substrate) contains fewer, more randomly dispersed actin-HMM complexes (Fig. 6). Occasionally, complexes are found deeper down in the cytoplasm near the nucleus.

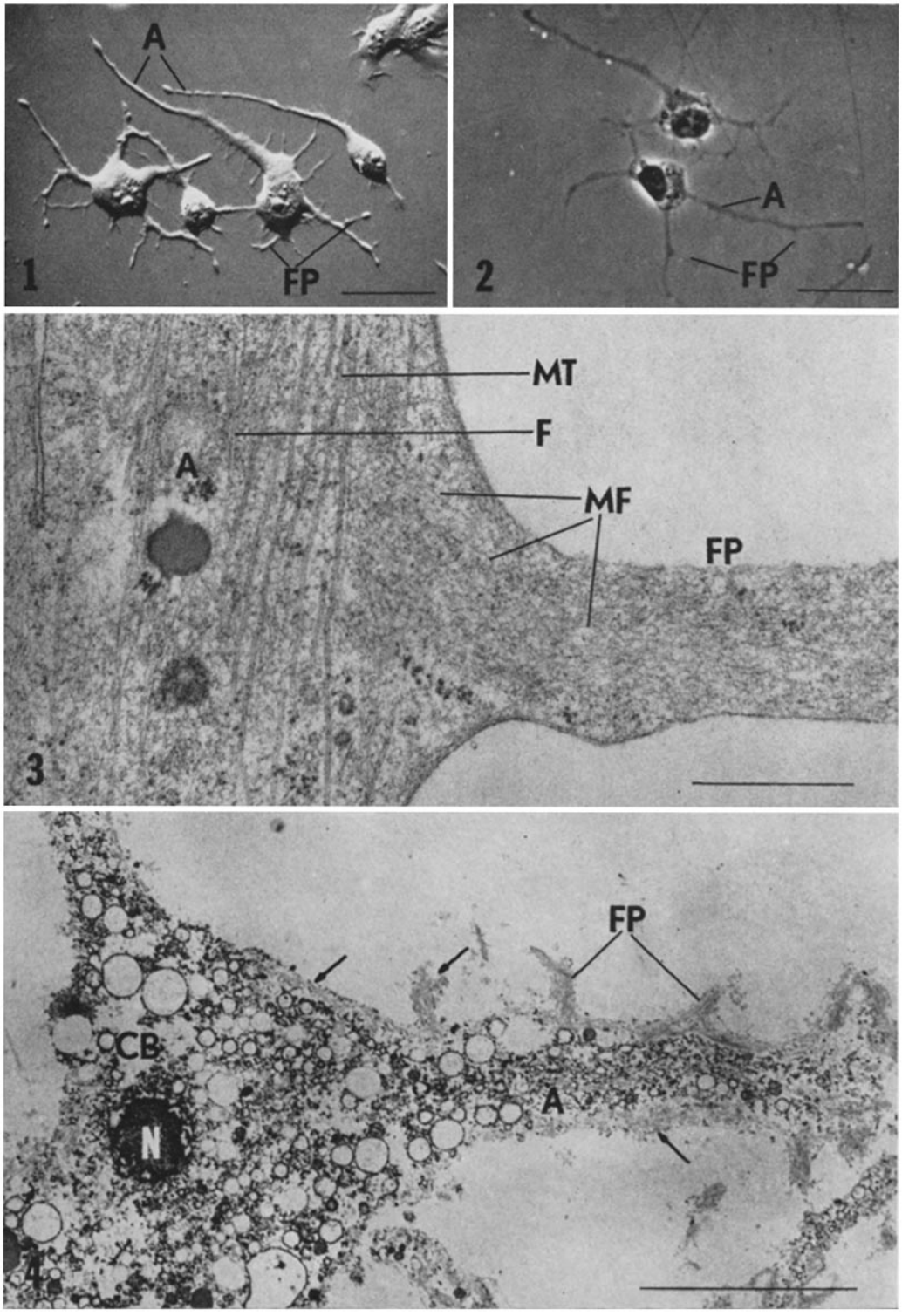
In axons, a layer of actin-HMM complexes appears localized beneath the cell membrane on upper, lower, and lateral surfaces. The majority of the complexes are oriented parallel to the long axes of axons. Local thickenings of the layer are observed throughout the entire length of axons. Groups of actin-HMM complexes running per-

FIGURE 1 Living neuroblastoma cells viewed with Nomarski differential intereference optics. Note axons (*A*) and filopods (*FP*). Scale line represents 50 μm. × 280.

FIGURE 2 Glycerinated cells which have been flat embedded in Epon 812. The overall shape of the cells is retained. Note axons (*A*) and filopods (*FP*). Inverted phase-contrast optics. Scale line represents 50 μm. × 310.

FIGURE 3 Electron micrograph of a thin section taken parallel to the long axis of an axon (*A*). Microtubules (*MT*) and filaments (*F*) are oriented along the long axis, while most microfilaments (*MF*) are seen in the filopods (*FP*). Scale line represents 1 μm. × 24,000.

FIGURE 4 A low magnification electron micrograph of a glycerinated, HMM-treated cell. The cell body (*CB*), nucleus (*N*), axons (*A*), and filopods (*FP*) are obvious. Arrows point to regions containing actin-HMM complexes. Scale line represents 10 μm. × 3,360.



pendicularly to the long axes of axons are often located in these thickened areas and usually continue into the filopod-like structures which project laterally from the axons (Fig. 7). Cross sections through similar groups of these complexes reveal an average diameter of 220Å (Fig. 7 *a*).

Glycerinated controls contain microfilaments in areas corresponding to the distribution of complexes in HMM-treated cells (Fig. 8). However, there appear to be fewer microfilaments when compared to the number of complexes seen after the addition of HMM, especially in axons and cell bodies. Microtubules are not stable with the glycerination procedure used, and therefore few are present either in experimental or in control cells. The filaments, however, appear unaffected by glycerination. Both filaments and the few microtubules which survive the glycerination procedure do not form complexes with HMM (Fig. 7). In addition, when 0.01 M sodium pyrophosphate (which dissociates actin-HMM complexes [7] is included in the HMM reaction mixture, no obvious actin-HMM complexes are formed (Fig. 9).

DISCUSSION

The *in situ* localization of actin-HMM complexes in neuroblastoma cells has already been demonstrated; however, the exact intracellular localization of the actin-like fibers has not been determined (4). Our short glycerination procedure preserves cell morphology and permits the detailed ultrastructural localization of actin-like components. Several major difficulties should be mentioned in interpreting the results obtained with this technique. The number of complexes seen in HMM-treated cells appears to be much greater than the number of microfilaments seen in controls or in cells fixed without glycerination. HMM could conceivably cause a transformation of G-actin present in the cells to F-actin (5), thus accounting for the increased number of complexes seen after HMM addition (see reference 7 for a detailed discussion of this possibility). The disappearance of microtubules following glycerination is not likely to account for the increased number of actin-HMM complexes, since it is improbable that HMM would interact with microtubule subunit protein in the same manner in which it interacts with actin.

The results of this study suggest that a network of actin-like protein is distributed continuously from the cell body to the nerve endings of a neuroblastoma cell. Although the contractile nature of HMM reactive fibers in nonmuscle cells has never been conclusively demonstrated, it is tempting to postulate that they are involved in cellular motile processes. The possibility of the existence of actin-like fibers in axons and their role in axoplasmic flow has been suggested by others (4, 6, 23). Recently, actin-like protein has been isolated from synaptosome-rich fractions, and its possible function in the release of synaptic transmitters has also been proposed (19). Our findings that actin-like fibers exist in both axons and their terminals provide the necessary morphological basis for the proposed function of actin-like fibers in axoplasmic flow and chemical synaptic transmission.

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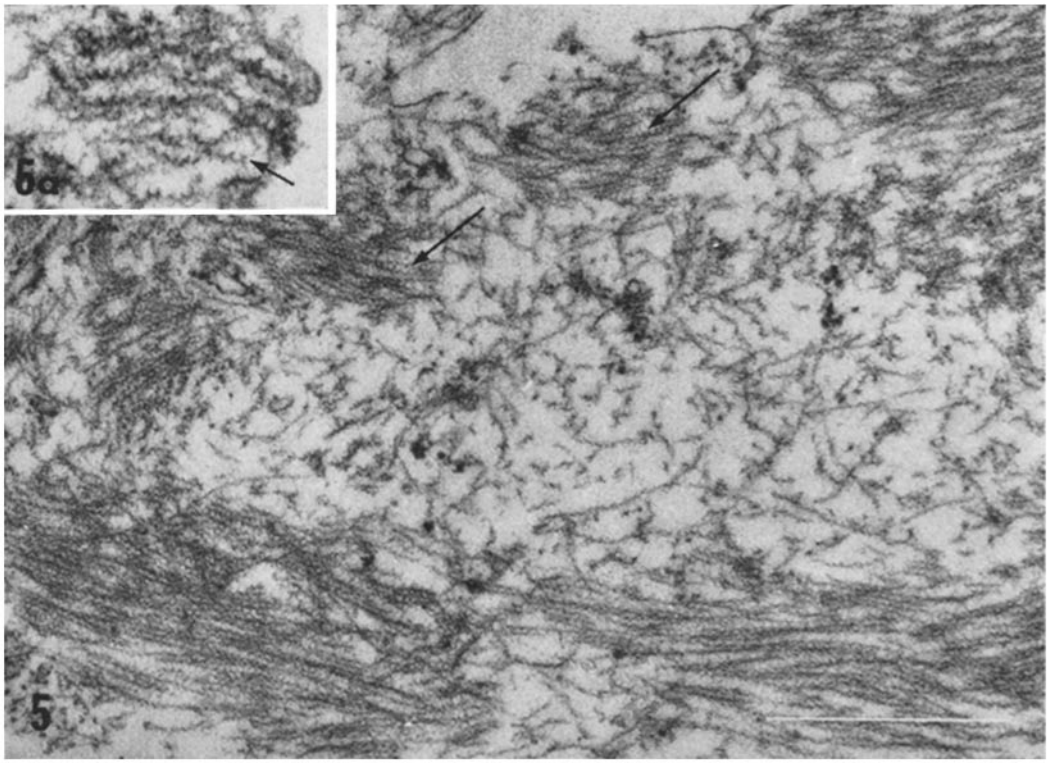
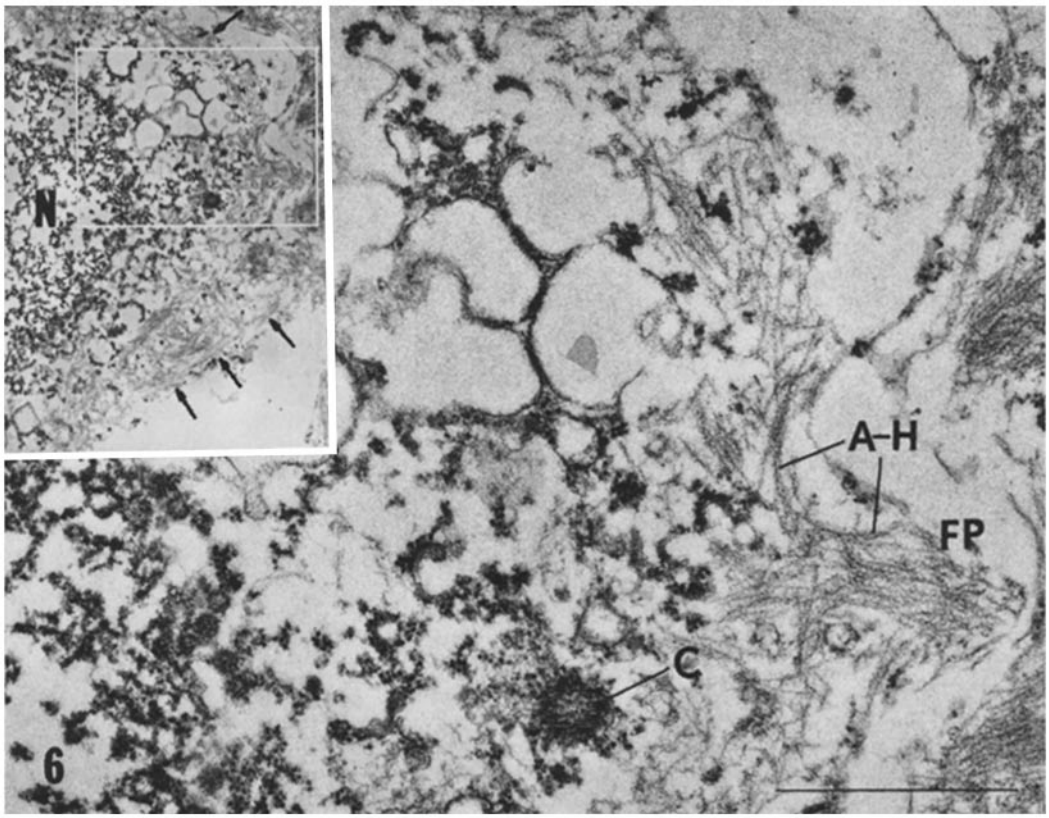
FIGURES 5 and 5 *a* Electron micrograph of a thin section taken just beneath the plasma membrane of the substrate side of the cell body. Masses of actin-HMM complexes are obvious (arrows). Arrowhead configurations are seen in Fig. 5 *a* (arrow). The scale line in Fig. 5 represents 1 μm and in Fig. 5 *a* represents 0.1 μm. Fig. 5, × 32,000; Fig. 5 *a*, × 64,000.

FIGURE 6 Low and high magnification electron micrographs of a thin section taken towards the upper surface of a cell body. The actin-HMM complexes are seen at the cell surface in the *insert* (arrows) and at higher magnification of the rectangular region (*A–H*). Note the centriole (*C*) and the base of a filopod (*FP*). *N*, nucleus. Scale line represents 1 μm. × 32,000; *insert*, × 7,200.





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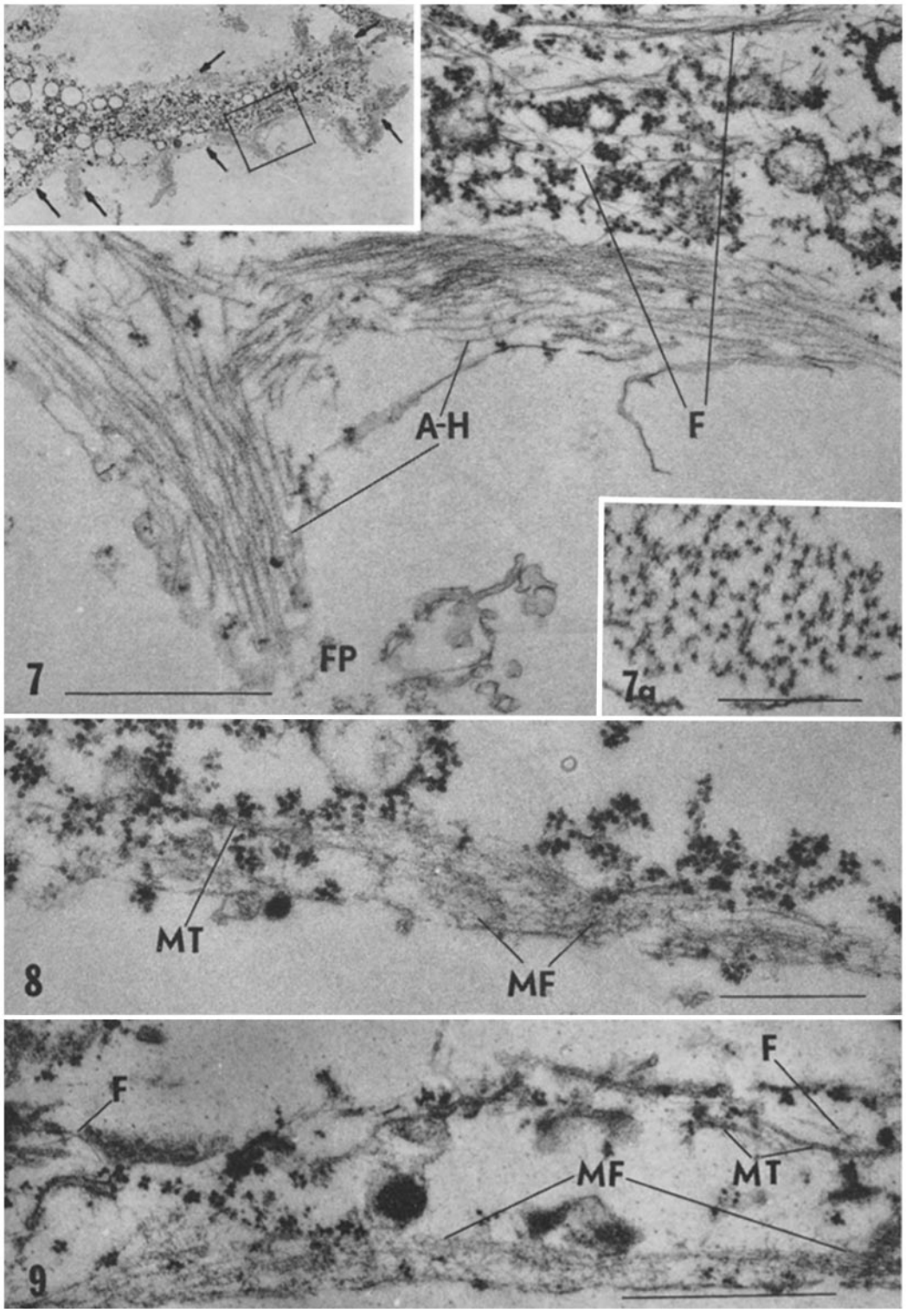
FIGURES 7 and 7 *a* Low and high power electron micrographs of an axon. Note regions containing actin-HMM complexes in the *insert* (arrows). Higher magnification of the rectangular region demonstrates that the complexes (*A–H*) are oriented along the long axes of both the axon and a filopod (*FP*). 100-Å filaments (*F*) do not interact with HMM. Scale line represents 1 μm. × 32,000; *insert*, × 2,300. Fig. 7 *a*: cross section of a group of actin-HMM complexes. The average diameter is 220 Å. Scale line represents 0.5 μm. × 44,000.

FIGURE 8 Electron micrograph of a thin section taken parallel to the long axis of an axon of a glycerinated cell not treated with HMM. Note microfilaments (*MF*) beneath remnants of the plasma membrane and a few microtubules (*MT*). Scale line represents 0.5 μm. × 44,000.

FIGURE 9 Electron micrograph of an axon treated simultaneously with HMM and 0.01 M sodium pyrophosphate. Note that most of the submembranous microfilaments (*MF*) retain their normal thickness and lack obvious decorations. *F*, filaments; *MT*, microtubules. Scale line represents 1 μm. × 33,000.



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